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IMMUNOLOGICAL ASPECTS OF MIGRATION AND TOLERANCE

Kurt Arkestål

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Immunological aspects of migration and tolerance

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Av
Kurt Arkestål

Huvudhandledare:
Docent John Andersson
Karolinska Institutet
Department of Medicine, Solna
Division of Immunology and Allergy

Bihandledare:
Senior Consultant Ola Winqvist
Karolinska University Hospital
Department of Clinical Immunology and Transfusion medicine

Fakultetsopponent:
Professor Thomas Giese
Universität Heidelberg
Department of Medicine
Division of Immunology

Betygsnämnd:
Docent Charlotte Dahle
Linköping University
Department of Clinical and Experimental Medicine
Division of Neuro and Inflammation Sciences

Docent Kenneth Jonsson
Uppsala University
Department of Surgical Sciences
Division of Orthopedics

Docent Helen Kaipe
Karolinska Institutet
Department of Laboratory Medicine
Division of Therapeutic Immunology

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ABSTRACT

The actions of the immune system are highly dependent on locality for development, memory imprinting, surveillance and effector functions. Migratory processes are therefore essential for maintaining immunological integrity of multicellular organisms. Given this complexity, it is perhaps not surprising that some of the functions employed by the immune system also are found in various other situations where a need for cellular motility is required such and angiogenesis and organogenesis.

To examine the complexity and distribution migratory markers we employed a hypothesis generating approach by investigating levels of chemokine receptors in different immune cell subsets in various tissues (Paper I and II). In order to evaluate how these markers change during development we focused our attention on bone marrow derived cells and compared them with mature leukocytes in bone marrow and peripheral blood. We were able to determine how different migratory markers change with increasing cellular maturity and were also able to determine differences between different patient groups.

We also addressed the expression of transcription factor Aire in bone marrow from both mice and humans (Paper III). In a series of experiments we examined expression pattern and regulation of genes related to Aire expression. We found that the transcription of *Aire* is present in peripheral dendritic cells in mice and down regulated in response to IFN-γ stimulation. We also demonstrated how Aire$^{-/-}$ KO mice have an altered composition of peripheral myeloid cells.

Finally, we investigated how IgE antibodies towards a carbohydrate epitope αGal arise in both patients with cat allergy and patients with macroparasitic infections (Paper IV). We showed how the two patient groups differ in serological profile and how such serological testing can be improved by using component based antigen techniques.

In summary, the thesis aims to contribute to how migratory capacity is altered with development and disease state by a combination of different laboratory and analytical techniques.

Till mormor och vän Carin Thelander-Looström

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”La clarividencia es el arte de ver lo invisible”

Gramática Española Moderna

LIST OF SCIENTIFIC PAPERS

I. Arkestål K*, Mints M*, Enocson A, Linton L, Marits P, Glise H, Andersson J, Winqvist O.
CCR2 upregulated on peripheral T cells in osteoarthritis but not in bone marrow
Scand J Immunol 2018 Oct 8:e12722.

II. Arkestål K, Enocson A, Winerdal M, Andersson J, Winqvist O
Global Assessment of Chemokine receptor expression on human myeloid bone marrow precursor cells.
*In manuscrip*t

III. Lindmark E, Arkestål K, Linton L, Adams W, Loré K, Enocson A, Winqvist O, Karlsson M.C.I. and Chambers B.J.
AIRE regulates genes important for development of dendritic cells in the bone marrow and is controlled by IFN-gamma
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IV. Arkestål K, Sibanda E, Thors C, Troye-Blomberg M, Mduluza T, Valenta R, Grönlund H, van Hage M
Impaired allergy diagnostics among parasite-infected patients caused by IgE antibodies to the carbohydrate epitope galactose-α1, 3-galactose
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*equal contribution

CONTENTS

LIST OF ABBREVIATIONS

APC	Antigen Presenting Cell
CCR#/CXCR#	Chemokine receptor #
BSA	Bovine Serum Albumin
HAS	Human Serum Albumin
PCR	Polymerase Chain Reaction
PAMPs	Pathogen-Associated Molecular Patterns
DAMPs	Danger-Associated Molecular Patterns
NOD	Nucleotide-binding Oligomerization Domain
NLR	NOD-Like Receptor
NOD mice	Non-obese diabetic mice
TLR	Toll-Like Receptor
PRR	Pattern Recognition Receptor
CMP	Common Myeloid Progenitor
CLP	Common Lymphoid Progenitor
HLA	Human Leukocyte Antigen
MHC	Major Histo-compatibility Complex
CD	Cluster of Differentiation
αGal	galactose-α-1,3-galactose
NFκB	Nuclear factor kappa-light-chain-enhancer of B cells
IRF	Interferon regulatory factor
ICAM	Inter-Cellular Adhesion Molecule
MCP-1/CCL2	Monocyte Chemoattractant Protein 1
TCR	T-cell receptor
ECM	Extra cellular matrix
ER	Endoplasmic reticulum
GPCR	G-protein coupled receptor
PIP_3	phosphatidylinositol (3,4,5)-trisphosphate
IP_3	inositol trisphosphate
PLC-β	phospholipase C-β
DAG	Diacylglycerol

T_{EMRA}	$CD45RA^+$ effector memory T-cells
T_{EM}	Effector memory T-cell
T_{CM}	Central memory T-cell
T_H	CD4 T helper cell
T_{FH}	Follicular T helper cell
T_{reg}	Regulatory T-cell
S1P	sphingosine 1-phosphate
S1PR1	sphingosine 1-phospate receptor 1
PSGL-1	P-selectin glyco protein 1
EGFR	epidermal growth factor receptor
TSA	tissue-specific antigen

1 INTRODUCTION

1.1 THE IMMUNE SYSTEM

The cells, molecules and organs that defend the organism from pathogens are commonly referred to as the immune system. It is composed of a wide variety of cells and structures that have coevolved with our environment and surrounding pathogens. Evolutionary pressure and generation time are helpful in understanding how the immune system has evolved. Many bacterial pathogens have a generation time as short as 20 minutes whereas humans can live for more than a hundred years. The passing of each generation introduces slight changes to the genome. As a consequence, a human of a hundred years faces 2.6 million generations of the same bacteria during a lifetime. Considering the endless possible genome combinations it is remarkable that the immune system can recognize and protect against potentially harmful evolutionary events.

The immune system also faces other challenges. Parasites have longer generation time than other microbial pathogens and one single parasite can persist several decades. Their genome size can be comparable with vertebrae species meaning they can employ more gene products that frequently are used to evade immunity. Thus, the immune system needs to be able to counter different types of pathogens. To this end certain the immune system has developed ways to identify and eliminate the potential treats. The way in which this is achieved depends highly on the branch of what is referred to as the innate immune system and the adaptive immune system

1.2 INNATE IMMUNITY

The first line of host defense consists of physical barriers like skin and mucosal membranes covering the body. These barriers prevent pathogens to establish a foothold by a combination of physical and chemical hindrance. As a wound opens, the innate immune system provides a second line of defense, protecting from further damage.

1.2.1 The myeloid linage

The innate immune system contains cells of myeloid origin and is the oldest and most conserved part of the immune system. Myeloid cells develop in the bone marrow from a common progenitor, CMP (Common Myeloid Progenitor) which in turn is derived from HSCs (hematopoietic stem cells). CMPs can give rise to polymorphonuclear cells, monocytes, macrophages and dendritic cells [1]. Polymorphonuclear cells, or granulocytes, mainly consists of neutrophils and to a lesser degree basophils and eosinophils. Granulocytes are short lived with a half-life of just a few hours. They make up roughly half of the leukocytes in circulation and are characterized by their segmented chromatin. Granulocytes act as first responders in case of infection to eliminate potential treats by ingesting pathogens, i.e. phagocytosis, and the release of inflammatory and anti-microbial mediators [2].

Monocytes develop in the bone marrow where they are transiently retained. The bone marrow continuous releases monocytes into the blood stream but during infectious stimuli; large amounts of monocytes can be released [3-5]. Released monocytes have different fates but most are short lived in circulation [6]. Some cells migrate into tissue to sustain the pool of tissue resident macrophages. Monocytes are divided into classical, non-classical and intermediate monocytes. A small portion of classical monocytes develop and mature into intermediate from which some differentiate into non-classical monocytes [6]. The major phenotypical difference between these types of monocytes is their surface expression of CD14 and CD16 [6]. All monocyte display phagocytic properties although classical monocytes are rapidly recruited to sites of injury [5] and display functional plasticity with low levels of proinflammatory cytokine production and perform antimicrobial phagocytosis as well as also promote healing [7]. Intermediate and non-classical monocytes are instead believed to patrol the vascular endothelium and produce large amounts of cytokines upon activation [8].

Macrophages are phagocytic cell present in parenchymal organs. They are specialized in phagocytosis but also perform specialized tasks of immune surveillance in the form of antigen presentation and cytokine production. Macrophages support neighboring cells and play a role during wound healing. The terminology M1 and M2 refers to the biased function of macrophages where the M1 macrophages are pro-inflammatory while M2 macrophages display healing and supporting properties [9]. Since macrophages have such diverse appearance and function in different organs they have often received distinct names in different organs (Kupffer cells in liver or microglia in the central nervous system). Although previously assumed, most tissue resident macrophages are not derived from monocytes but instead from cells derived from the yolk sac during fetal development [10]. However, monocyte derived macrophages can in many cases replace tissue resident macrophages albeit this appears to be less common than previously assumed [10].

Dendritic cells comprise a diverse group of cells with distinct ontogeny from monocytes but like macrophages and dendritic cells can be derived from monocytes, especially during inflammatory conditions [11]. Human dendritic cells can be dived into plasmacytoid DCs (pDCs) and myeloid or conventional DCs (mDCs or cDCs). Plasmacytoid DCs differ from myeloid DCs both in function and appearance with eccentric nucleus and a prominent ER folding as well as the production of class-1 interferons [12]. pDCs are dependent on transcription factors IFN8 and BATF3 and lack many myeloid markers. Their main function appears to be support of other antigen presenting cells and to convey antigen presentation of viral antigens. cDCs can in turn be subdivided into two subcategories termed cDC1 and cDC2. The phenotypic difference of these subset is not completely understood but cDC1 are also dependent on IFN8 transcription factor and display chemokine receptor XCR1. cDC2 is the most monocyte like DCs with retained CD14 expression and are dependent on RELB and IRF4 transcription factor [11] for development. cDCs are highly specialized antigen presenting cells and fundamental to bridge the innate and the adaptive immune system.

1.2.2 Innate pattern recognition

Although the peripheral circulation is continuously patrolled by primarily circulating monocytes and neutrophils, some myeloid cells are tissue resident [13]. Macrophages and dendritic cells are primarily located at sites of high pathogen exposure such as the epidermis or the submucosal epithelium in the gastrointestinal or respiratory tract where they serve as *"sensor cells"*. Sensor cells monitor their surrounding and identify potentially harmful pathogens mainly through pattern recognition receptors (PRRs) which recognize evolutionary preserved molecular pattern in microbes referred to as pathogen-associated molecular patterns (PAMPs). PRRs can detect extra-cellular presence of PAMPs such as bacterial flagelin or lipopolysaccarid (LPS) via Toll-like receptors (TLRs), or intracellular bacterial presence via NOD-like receptors (NLRs) [14]. PRRs also recognize danger associated molecular patterns (DAMPs) which constitute molecular components released during tissue damage such as extracellular nuclear material or mitochondria. There are many distinct PRRs, however, their downstream receptor signaling typically entails NFκB and IRF transcription factors.

As consequences of NFκB and IRF transcription, a cascade of cytokines and chemokines are synthesized and released which further activate bystander cells. Chemokines together with now activated endothelial cells expressing selectins attract further leukocytes such as additional neutrophils and monocytes. Activated myeloid cells such as macrophages and dendritic cell phagocyte pathogens and debris from the site of inflammation. In the phagocytic process of endocytosis, the plasma membrane encircles the pathogen and the engulfed material is internalized in vacuoles referred to as phagosomes. The acidity of the phagosome kills off the pathogen. The phagosomes then fuses with another vacuole termed lysosome containing lytic enzymes degrading pathogen-derived material even further[14]. The pathogen derived peptides residues are eventually presented on the cells surface via HLA class II (Human Leukocyte Antigen) or MHC class II (Major Histocompatibility Complex). Cells expressing MHC-II are also referred to as professional antigen presenting cells (APCs). The APC migrate towards the T-cell zone of the draining lymph node. This migration follows a gradient of CCL19 and CCL21 expressed by fibroblastic reticular cells in the lymph node attracting APCs and naïve T-cells expressing the chemokine receptor 7 (CCR7) [15, 16].

1.2.3 Antigen presentation

Most infections will be cleared by the innate immune system. However, the innate immune system has no memory and will respond in the same manner every time a pathogen reoccurs. This poses a challenge since the pathogen may as previously described have undergone several hundred generations of cell division introducing new ways of sidestepping the innate defenses. In order to counteract this and maintain an effective response, the innate immune cells present antigens to T-cells thus activating the adaptive immune system that confers specificity and memory.

HLA class II molecules are with some limitations capable of presenting an array of peptides, usually between 13-17 amino-acids long. Remarkably, each T-cell express approximately 30000 copies of the same T-cell receptor (TCR) specific for a presented peptide in the context of the HLA class II molecule. This is referred to as HLA class II or MHC-II restricted antigen presentation. HLA class II presentation is mainly restricted to present phagocytosed extracellular antigens [17].

Importantly, the APC has to provide a secondary signal indicating whether the antigen poses a threat or not. If no such secondary signal is provided, the T-cell will become unresponsive even if the TCR recognizes the antigen perfectly. In contrast, if the antigen is recognized by a naïve T-cell in a pro-inflammatory cytokine milieu with DAMP or PAMP signaling, a costimulatory secondary signal (CD80/CD86) provided by the APC to activate the T-cell and begin clonal expansion [18]. Additionally, a third signal consisting of pro-inflammatory cytokines are required to initiate the adaptive immune system [19].

Another important form of antigen presentation is provided by HLA class I present on all nucleated cells. HLA class I presents 8-11 amino acids long peptides derived mainly from cytosolic proteins. Presentation of cytosolic antigens serves a purpose both in the case of intracellular pathogens such as viral pathogens and intracellular bacteria but also during neo-antigen present during malignancies [18].

In a special case of antigen presentation, mainly cDCs engulf large volumes of extracellular fluid containing debris from dead cells and soluble peptides of pathogen origin in a process called micropinocytosis. These proteins then escape the phagolysosome entering the cytosol. Thereby, extra and intra cellular pathogen derived peptides may be presented by the HLA class I which is referred to as cross presentation [20].

1.3 ADAPTIVE IMMUNITY

The adaptive immune system is as the name suggest capable of adapting to challenges posed and to modify immune response if needs be. It is evolutionary much younger than the innate counterpart but highly conserved within vertebrate species indicating its' importance for complex organisms [21].

1.3.1 The lymphoid linage

The lymphoid linage is derived from the common lymphoid progenitor (CLP) which like CMP is derived from HSC and comprises primarily T and B cells of the adaptive immune system. Cells of the adaptive branch of the immune system are able to recognize specific antigens through their respective receptor termed B and T cell receptors. These receptors share many characteristics in that they are not encoded in the germline genome but instead are the result of a process termed somatic recombination. During somatic recombination, the antigen binding receptor is assembled from a variety of gene segments. Both the B- and T-cell receptor is composed from gene segments consisting of a variable domain (V), diversity domain (D) and joining domain (J). Various versions of these gene segments are randomly assembled to form the antigen binding receptor in what is commonly known as V(D)J rearrangement or somatic recombination. The process of somatic recombination is highly conserved trough evolution and governed by a few essential enzymes. Interference in this recombination process can cause severe combined immune deficiency (SCID) with negligible levels of adaptive immune cells [22]. Through this process the number of potential antigen binding receptors widely exceeds all potential protein products of the germline genome.

1.3.2 T-lymphocytes

Thymus dependent lymphocytes or T lymphocytes a derived from the bone marrow from CLP but undergo selection in the thymus. T–cells precursors called thymocytes egress from the bone marrow niche through a process which is not fully understood but sphingosine 1-phosphate (S1P) has been implicated [23, 24]. The thymocytes lack many of the characteristics of mature T-cells including their antigen binding receptor (T-cell receptor, TCR) and either of two co-receptors termed CD4 and CD8. When thymocytes enters the thymic cortex their TCR start to undergo somatic recombination in a multistep process. If the thymocyte fails to form a functioning T cell receptor the cell will perish. Upon forming a T-cell receptor the cell starts to express both the CD4 and the CD8 co-receptor. The T-cell now needs to receive stimulus through the newly formed TCR for continued survival. This is achieved through interactions with thymic stroma cell which express HLA class I and HLA class II by which it present self-antigens. TCRs recognizing HLA together with self-antigens provide survival signals while those cells that fail to recognize HLA succumb to apoptosis. This process of testing TCR function is referred to as positive selection. In the medullae T-cells whose TCR form too strong of an attachment to the HLA containing self-peptides presented by medullary epithelial cells (mTEC) are also deleted in a process called negative selection. These cells could otherwise become self-reactive ie. perceive the body's own proteins as pathogen-derived causing autoimmunity. Many proteins are tissue specific and are normally only present in their respective organ. One such example is insulin produced β-cells in pancreatic islets. Consequently, the immune system has had to devise strategies to allow for negative selection of T cell reactive against such proteins. To this end the thymus produce a number of tissue-specific antigens (TSA) through the transcription factor autoimmune regulator (AIRE) in mTECs [25]. We will return to AIRE in the tolerance chapter.

1.3.2.1 CD4 and CD8

Depending on which class of MHC molecule the T-cell primarily has interacted with in the thymus it will express either of the two co-receptors CD4 or CD8. The TCR is what is called MHC restricted meaning it does not recognize antigens outside the context of the MHC presentation. As previously mentioned, MHC-I is expressed on all nucleated cells, presenting cytosolic peptides. The CD8 co-receptor only interacts with MHC-I and therefore CD8 T-cells can only recognize MHC-I presented peptides. In turn, MHC-II is expressed on APCs and only T-cells expressing the CD4 co-receptor will recognize antigens presented on MHC-II [26].

During thymic negative selection, self-reacting T-cells are deleted. However, a subset of T-cells recognizing MHC/self-antigen is believed to be the result of a relatively high affinity binding of their TCR to MHC/peptide complexes yielding a subset of T cells termed regulatory T cells (T_{reg}) which in contrast to normal T-cells inhibit inflammation [27].

T cells leave the thymus committed to either of the CD4 and CD8 subset. These cells express the surface marker CD45RA indicating their naïve phenotype as they have yet to encounter their cognate antigen in the periphery [28]. Naïve T cells will remain in circulation between the T-cell areas of secondary lymphoid organs and the blood searching for an APC presenting their cognate antigen.

1.3.2.2 T-cell priming

Once naïve T-cells encounter their cognate antigen presented by MHC together with the appropriate stimulatory co-receptor and a pro-inflammatory cytokine milieu they become activated and begin to undergo rapid cell division. The progeny is referred to as effector cells and express CD45RO. During this process the cells down regulate their expression of CCR7 and CD62L and instead express S1PR1 which enable the cells to enter the efferent lymphatics and subsequently the blood circulation due to the higher concentration of S1P [29]. The newly formed effector cells perform different task depending on which co-receptor is expressed.

1.3.2.3 T-cell subsets

The CD8 and CD4 co-receptor interacts with different MHC class why these subtypes have different target cells. The CD8 subset respond to MHC-I expressed on all nucleated cells and its' function is to induce apoptosis or kill any cells which present antigens associated with intra cellular pathogens like viruses. The CD8 subset is because of this often referred to cytotoxic T-cells as they induce cell death of infected cells.

The CD4 subset, referred to as T-helper cells is more diverse than the CD8 subset. T-helper cells direct immune responses and can assist other cells in intracellular killing of phagocytosed pathogens or induce class switching in B-cells. The different T-helper subsets also secrete cytokines which can both amplify and suppress immune responses.

1.3.2.4 T-cell phenotyping based on chemokine receptor expression.

CD4 T-cells consist of several subsets. Interestingly, it has become apparent that T helper cell subsets, defined by their cytokine production, exhibit distinct signatures of chemokine receptors[30]. Thus it has become increasingly common to phenotype lymphocyte subsets based on their chemokine receptor expression[31]. Naïve T-cells home to secondary lymphoid organs they express CCR7, CD62L and CD45RA. Some effector subsets referred to as central memory T cells (T_{CM}) also home to lymph nodes and express CCR7, CD62L. Since they have encountered their antigen they express CD45RO. T_{CM} express low levels of cytokines and are believed to represent a pool of primed T cell with propensity for subset specific cytokine production upon antigen reencounter [32]. Other effector T-cell done not home to secondary lymphoid organs and is hence devoid of CCR7 and CD63L expression but instead express chemokine receptors for tissue homing. For example, CCR7^{-} CD45RO is referred to as effector memory T-cells or T_{EM} [33]. It was previously believed that T_{EM} in some cases revert to a naïve-like phenotype expressing CD45RA in the CD8 compartment termed T_{EMRA} [33]. However, the T_{EMRA} have been shown to terminally differentiated [34].

Distinct CD4+ T-cell subsets also differ in their ability to produce cytokines and facilitate immune responses depending on the type of pathogen they have encountered. The T_H1 cells secrete IFN-γ and other cytokines which assist phagocytic cells in intracellular killing of pathogens while T_H2 cells secrete IL-4 secreted to evoke a class switch by B-cell to produce IgE important for defence against macroparasites. T_H17 cells instead produce IL-17 important for neutrophil driven responses important in fungal infections. These subset where initally defined by their cytokine production although it has become increasingly accepted that these cells also differ in chemokine receptor expression. In this manner $T_H1$cell has been shown to preferentially express CXCR3 while T_H2 express CCR4 and $T_H17$express CCR6 [35].

1.3.3 B-lymphocytes

B-cells, like T-cells, are adaptive immune cells that also undergo V(D)J gene rearrangement. The formation of the B-cell receptor (BCR) vary slightly from that of the T-cell receptor in that the B-cell receptor will undergo second turn of somatic recombination if the formation of the receptor is unproductive. Unlike the TCR, which undergo development and positive and negative selection in the thymus, BCR is not MHC restricted. Thus, B-cells do not undergo positive selection which instead increases the necessity for negative selection to delete self-reacting clones. If the BCR bind antigen in the bone marrow the B-cells can undergo additional gene rearrangements to reform the BCR in order to escape deletion. As the BCR is formed the B-cell exit the bone marrow although not fully mature. The B-cell will undergo further maturation is now exposed to all self-antigens in the periphery. If its cognate antigen is encountered without the presence of inflammation the cell will perish. Immature B-cells eventually migrate to the spleen where they compete to enter B-cell follicles where they will mature into functioning B-cells.

1.3.3.1 B-cell priming

The naive B-cell will migrate to the B-cell follicles of lymph nodes or the spleen but instead of using CCR7 naïve B-cell express the chemokine receptor CXCR5. The B-cell follicle is constantly exposed to antigens brought in by the afferent lymphatics and contains follicular dendritic cell and marginal zone macrophages that capture incoming antigens. Rather than degrading the antigens, they are instead presented intact to naïve B-cells. Binding of an antigen will cause internalization of the BCR:antigen complex whereby the antigen is degraded and presented via the MHC-II. When naïve B-cells encounters their antigen they begin to express CCR7 migrating toward the T-cell zone. The B-cell needs further support from a follicular T helper cell (T_{FH}) to become activated. The T_{FH} is a subset of CD4 T lymphocytes that instead of leaving the secondary lymphoid organ upon priming migrate toward the B-cell follicle by induced expression of CXCR5 and thereby co-localizing the two in the marginal zone of the B-cell follicle. Here the B-cell present the antigen to the T-cell via the MHC. If the T_{FH} recognize the antigen the B-cell presents it will get licensed by the T-cell to become activated and begin clonal expansion and further differentiation. Additionally, B-cells can be activated in a T-cell independent manner as a result of multivalent BCR bonding causing crosslinking of adjacent BCRs. Since the antigen needs to display repetitiveness it is common that these antigens constitute polymers found in bacteria, plants or parasites.

1.3.3.2 Affinity maturation, antibodies and class switch

The B-cell receptors have a similar antigen binding structure as the T-cell receptor and similarly have the capacity to bind virtually any antigen. It is made up of two heavy chains and two light chains to form a “Y”- like structure. While the TCR is in need of proximity to exert effect through the TCR, the BCR can be secreted in a soluble form of the B-cell receptor termed antibody. Whereas the variability and specificity of the B-cell receptor is due the same V(D)J gene rearrangement as the T-cell receptor, the antigen binding site can further point mutate to strengthened affinity for the antigen can be formed in a process known as somatic hyper-mutation or affinity maturation [36].

Naïve and newly activated B-cell express antibody class IgM and IgD. During maturation B-cells form germinal center in which they are continually dependent on support from T_{FH} and follicular DCs. As B-cell become activated and initiate maturation to antibody producing plasmablasts or plasma cells several changes are introduced to the B-cell receptor. This includes changes to both the antigen binding domain and the heavy chain Fc domain. The changes of the antigen binding domain are cause by introduction of mutation during mitosis during a process known as affinity maturation. High mutation rates together with a need for continued BCR signaling cause an increased affinity for the antigen. Additionally, CD40L expression and cytokines produced by the T_{FH} may cause genomic excisions causing alterations in the Fc portion of the heavy chain gene by which the antibody class is altered. Antibodies can be produced in varietiesor classes, which have different effector mechanisms. This allows class switching to provide an ability to adjust immune responses depending on the pathogen.

1.4 TOLERANCE

The immune system needs to act swiftly and eliminate treats rapidly before an infection poses a systemic challenge. Simultaneously, the immune response needs to be balanced and not cause harm or spread inflammation beyond the infected area. The concept of tolerance is that the immune-response is controlled and limited to actual harmful pathogens while not reacting against healthy tissue or other non-pathogenic material such as animal dander or gut-bacteria i.e. discrimination between self and non-self. As described above, the innate immune system needs microbe and virus specific PAMPs to initiate an inflammatory response. However, as the innate immune system has no memory it can by itself not judge whether an antigen is pathogen derived or poses a self-antigen from the affected tissue or even harmless non-self-antigen such as foodstuffs or animal dander. The adaptive branch is therefore needed to control and dampen an inflammatory response.

1.4.1 Central Tolerance

The term central tolerance refers to processes during development in primary lymphoid organs (ie. bone marrow and thymus) that renders the immune system non-responsive alternatively actively suppressing inflammation[37].

1.4.2 Central T-cell tolerance

As previously described, both the B- and the T-cell receptor are assembled by somatic recombination to generate a diverse repertoire of receptors. For central tolerance, positive and negative selection is of outermost importance. The presentation of self-antigens in the thymus forms the basis of T-cell mediated tolerance. The TCR is tested repeatedly for ability to recognize self-peptide:MHC during thymic development and clones with whose receptor bind too strong are deleted. Many antigens are tissue specific and intuitively not expressed in the thymus, which would make it impossible to negatively select TCRs recognizing these self-antigens. However, the transcription factor AIRE, present in medullary thymic epithelial cells (mTEC), drives ectopic expression of such self-antigens for the purpose of negative selection of T cells carrying self-recognizing TCRs. The importance of AIRE based transcription is demonstrated in monogenetic disorder in which mutations in the *AIRE* gene termed severe autoimmune conditions known as autoimmune polyendocrine syndrome – 1 (APS-1). The clinical symptoms vary but commonly include skin symptoms with vitiligo, alopecia and cutaneous candidiasis associated with endocrine disorders like diabetes mellitus hypoparathyroidism and Addison's disease [38].

T cells that have successfully recombined their TCR and that do not display a sufficiently high affinity for self-peptide:MHC complexes to be negatively selected, will form the pool of mature T cells. T cells that escape negative selection with a relatively high affinity for self-peptides develop into immunosuppressive T cells known as regulatory T cells (T_{reg}). T_{reg} developed in thymus are referred to as thymic T_{regs} (tT_{reg}). T_{regs} are antigen specific but instead of mediating inflammatory responses they dampen the inflammation.

1.4.3 Central B-cell tolerance

Central tolerance among B-cells is less well characterized. In contrast to the TCR, the BCR does not undergo positive selection and therefore bind unprocessed antigens outside the context of the MHC presentation. Hence, the newly formed BCR could potentially bind antigens directly after forming. Therefore, binding of any antigen during bone marrow development signals self-antigen. Unlike T-cells, B-cells can undergo a second round of somatic recombination of the BCR light chain. If the resulting second BCR is unproductive or remain self-reactive the clone will be deleted. B-cell egress from the bone marrow in an immature, transitional state with a fixed BCR. Cells are now exposed to most antigens through circulation. If the BCR encounter its antigen without presence of proinflammatory signaling, the clone will be deleted or anergic. The immature B-cells need to reach the spleen for continued development in to mature B-cells.

1.4.4 Peripheral Tolerance

Although T-cells undergo positive and negative selection in the thymus and B-cell undergo a similar process in the bone marrow some self-reactive lymphocytes escape during the selection process. Thus there is a need to control these potentially harmful cells by peripheral tolerance mechanisms in the periphery.

Peripheral tolerance occurs in mature lymphocytes which have a fully developed specific antigen receptor. Adaptive immune cells need co-stimulation to become activated. Cells that encounter their antigen without the presence of co-stimulation turn unresponsive or anergic. Anergy is believed to be one of the most important forms of peripheral tolerance and develop in absence of activated dendritic cells. However, during an ongoing inflammation, antigen presenting cell display foreign- and self-antigens indiscriminately. There is therefore a potential risk of self-antigens being presented in a manner that induces T cell activation.

One way in which this is avoided is the presence of thymically derived T_{reg} cells (tT_{reg}) which when presented self-antigen dampen the immune response by secreting IL-10 and TGFβ. TGFβ can also induce naïve T-cell to develop into peripheral regulatory T cells (pT_{reg}). These cells serve a similar function as tT_{reg} although display a less stable phenotype. Tregs also suppress immune activation by having a high expression of the IL-2 α-chain CD25 why stimulating IL-2 is sequestered from effector cells in the area. Finally, Treg cells have the ability to use CTLA-4 to suppress the activation of APCs. The T_{reg} phenotype is under the control of the transcription factor FOXP3 and patients who display mutations in the *FOXP3* gene display severe autoimmunity in a syndrome of immune-dysregulation, polyendocrinopathy, enteropathy and X-linked(IPEX) [39]. The generation of tT_{reg} in the thymus is dependent on AIRE transcription for the production of self-antigens. Although previously contested, multiple examples of peripheral expression of AIRE as well as in bone marrow have been described. Several findings suggest that peripheral tolerance is affected in Aire deficiency. Immunized $Aire^{-/-}$ mice with an exogenous antigen T cells display a hyperproliferative response [40]. Thus, Aire may participate in the peripheral regulation of T cell activation. The expression of AIRE in humans and mice have been found in different myeloid cells in various tissues involved in regulation of immune responses such as in the thymus, spleen, lymph nodes and in the bone marrow [41]. *AIRE* mRNA has also been found in human B cells and monocytes in peripheral blood [41, 42]. The notion that Aire contribute to peripheral tolerance has gained traction following studies showing functional properties of Aire expression in the periphery [43].

B-cells are less dependent on proximity for their adaptive response due to their ability to secrete soluble B-cell receptors (antibodies). Peripheral B-cell tolerance is believed to be induced if the naïve B-cell already has encountered its antigen in the periphery and bound it upon entering the T-cell area of the lymph node which in turn renders the B-cell anergic which leads to deletion of the self-reactive B-cell clone. The B cell typically needs to be licensed for switching and activation by a $CD4^+$ T helper cell, in agreement with the target antigen. Thus, B cell tolerance is here mediated by T cell tolerance.

1.5 LOSS OF TOLERANCE

Loss of tolerance to harmless non-self-antigens is commonly known as allergy and such antigens are commonly present in plant pollen or animal dander. The typical allergic reaction of itching, redness, swelling and mucosal hypersecretion is mediated by the IgE class antibody [44]. The IgE antibody binds the allergen and the Fc receptor bind to FcE receptor on mast-cells and basophils releasing a cascade of histamine causing the inflammatory symptoms. Class swich to IgE is induced by IgE response is intended for combating parasitic infections and IgE titers are high in parasite infected patients [44]. Apart from causing hypersensitivity to plant and animal derived carbohydrates and proteins the allergic reaction may also be aimed at foodstuffs or therapeutic drugs .

Loss of tolerance towards self-antigens also cause autoimmune disorders. The spectrum of autoimmune disorder ranges from tissue specific as in the case of Addison disease or type 1 diabetes to systemic in the case of SLE, depending on antigen location. The exact cause is many cases unknown but in many autoimmune disorders there is a strong link to HLA type indicating that certain MHC molecules are more prone to present self-antigens leading to autoimmunity. The clinical symptom often arises after a challenge to the immune system such as injury or infection.

One disorder which bridges the gap between allergy and autoimmunity is celiac disease where the ingested protein gliadin attaches to self-antigen in form of the enzyme transglutaminase. The protein complex is then presented together on APC giving rise to antibodies directed both towards gliadin derived peptides as well as auto-antibodies towards transglutaminase[45].

1.5.1 Macroparasite infections

Macroparasites form a diverse group of multicellular eukaryotes which have the commonality that they are dependent on substrate from another organism like humans for survival or reproduction. The group includes insects and helminths as well as schistosoma species. Macroparasites pose a very different challenge to the immune system than viral and bacterial antigens. Firstly, macroparasite are typically carry a diploid genome meaning they can replicate through sexual reproduction unlike viruses and bacteria, Second, their spans over longer time periods and may persist for many years in the same host. Third, their size is in many cases huge compared to other challenges that the immune system faces.

Despite their size insect, helminth and schistosoma infections often cause very limited inflammation and does not elicit adaptive nor innate immune responses. To counteract macroparasitic infection, T_H2 cells elicit responses by secreting IL-4. The way in which parasites are initially recognized by the innate immune system is not fully understood but several PAMP signaling receptors, including TLR-2, has shown to recognize chitin [46]. Chitin is a polysaccharide which is present in exoskeletons of insects, schistosoma species and helminth eggs in the form of polymers. The T_H2 response induced by macroparasitic infections are characterized by IgE antibody production. The IgE antibody elicit a release of histamins and lyctic enzymes when the Fc portion of the receptor crosslinks on mast cells and basophils thus causing a strong local immune response of vasodilatation, itching and smooth muscle contraction which together are believed to help in physically expulse the parasite.

As macroparasites have a large genome and as eukaryotes they are able to mimic or even alter glycosylation patterns of the host [47, 48]. Many macroparasites are host specific and are unable to live or replicate outside the human body. Given their long lifespan, many macroparasites have to employ completely different strategies for survival and immune evasion. An interesting example of parasite-host interaction is in how *Leishmania* species induce a T_H2 response, thereby avoiding a T_H1 response which for the parasite would be detrimental [49].

1.5.2 Allergy

The term allergy describes a state of immunological memory towards an otherwise harmless antigen which should not evoke an immune response. Antigens which are commonly cause allergies are found in animal dander, food stuffs or pollen and are referred to as allergens. Symptoms in allergy are commonly wheezing, rhinitis, irritation, swelling, redness and hypersecretion.

The prevalence of allergy has increased with improved living standards this has been attributed to the so called hygiene hypothesis [50] were a diminished infection burden during childhood result in a general T_H2 skewing resulting in allergic symptoms [51, 52]. The T_H2 skewing of immune responses seen in allergy is also seen in macroparasitic infections and several studies have therefore suggested that the absence of parasitic infection in modern society could explain the increase in allergy prevalence [53, 54]. Indeed, eradication of parasitic infections has shown to increase prevalence of allergic symptoms [55]. However, the prevalence of allergic disorders within western society has not continued to rise over the last decades and although the hygiene hypothesis does provide correlation with the rise in allergic symptoms it does not provide proof of causality of allergic symptoms [56].

1.6 CELLULAR TRAFFICKING

The migratory system and the chemokine receptors described above are essential for proper development and function of the immune system. Immune cells are in many cases highly mobile and serve their function in different localities from where they developed. The migratory machinery employed by immune cells is also used by other cells that depends on trafficking and migration. For example chemokine receptors partially govern organogenesis during fetal development and the formation of new blood vessels.

As previously described, myeloid sensor cells can be produced in the bone marrow but primarily serve their function adjacent to mucosal barrier of the gastrointestinal tract, airways and skin where infections are likely to occur. The development and function of adaptive immune cells require timely cell trafficking to enable co-localization of antigen presenting cells and naïve or memory type T-cells

There is a constant circulation of immune cells to and from mucosal barriers, tissue, bone marrow and other lymphoid organs. Essential for this cellular trafficking are a set of selectins, integrins and chemokines which together govern cell motility.

1.6.1 Chemokines and their receptors

There are more than 50 known chemokines and approximately 20 chemokine receptors yielding a certain redundancy in the system with an overlap of chemokine receptors binding several chemokines [57]. The nomenclature of chemokines and their receptors has been standardized and are now classified as C, CC, CXC and CX_3C based on the amino- terminal positioning of cysteine residues [58]. However, many older names and acronyms are still used why the nomenclature can be confusing.

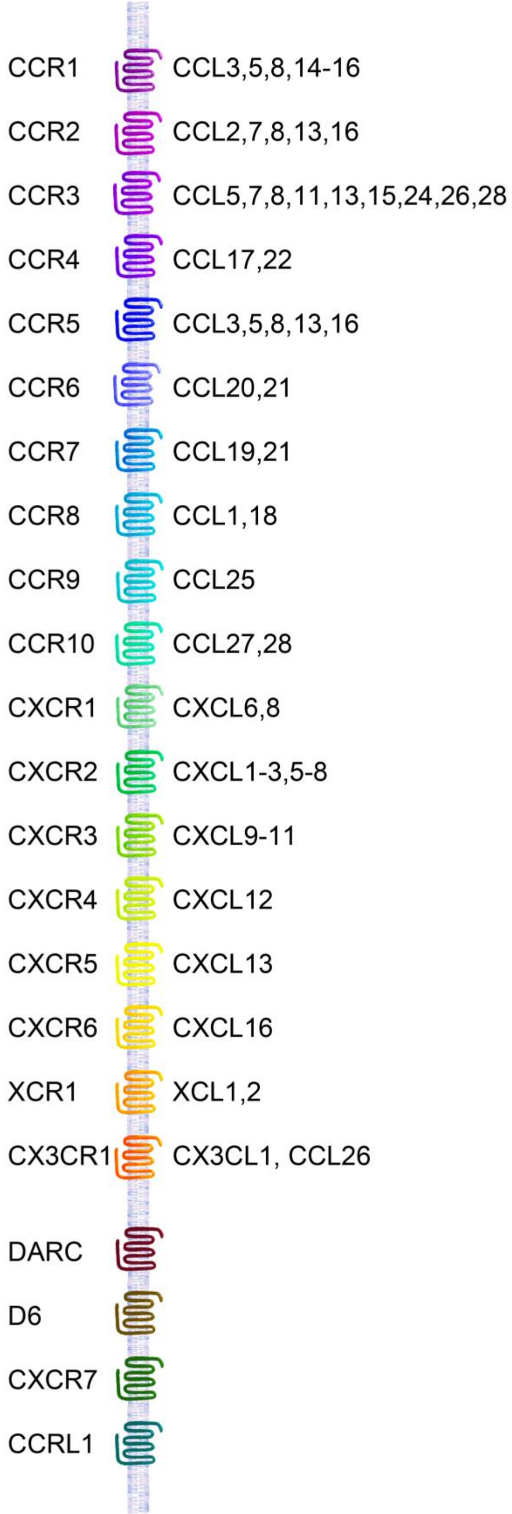

*Fig 1.Chemokine receptor and associated ligands displaying overlapping receptor-ligand affinity***.** *Adapted from Griffith and colleagues, 2014 Courtesy of Dr Malin Winerdal*

1.6.2 Downstream regulation of chemokine receptor signaling

Chemokine receptors belong to the seven transmembrane G-protein coupled receptor family (GPCR). The human genome carries some 800 GPCR genes of which 108 are drug targets encompassing some 34% of all FDA approved drugs [59]

The GPCR downstream signaling has proven complex where several downstream signaling pathways are known. Generally, the intracellular domain of the receptor bind a heterotrimer of G-proteins (α, β, γ- subunits) during resting conditions.

Binding of a receptor ligand will induce conformational changes of the intracellular domain of the receptor dissociating the α-monomer and the βγ-dimer exposing the G-protein binding serine or threonine residues. Both the Gα and the Gβγ subunit may induce downstream signaling but in the case of chemokine receptors the Gβγ subunit is essential for migration.

The Gα and the Gβγ subunits both induce second messengers such as phospholipase C-β (PLC-β) which convert the phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) to inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 cause release of calcium from endoplasmic reticulum (ER) and DAG in turn induce protein kinase C (PKC) which inactivate the GPCR by phosphorylation [60]. GRKs are also induced and may also phosphorylate the GPCR in an inhibitory feedback loop.

PKC or GRKs readily phosphorylates the intracellular serine or threonine residues in the cytoplasmic tail of the ligand bound GPCR rendering the receptor inactive. The phosphorylated receptor readily binds β-arrestins often terminating in receptor internalization acting as a signal modifier at the cell surface level [61]. These β-arrestins bind the same sites as the Gα-subunits following phosphorylation and thereby compete for these binding sites. Different ligands can therefore induce so called biased agonism were intracellular signaling pathways vary depending on the ligand [62].

1.6.2.1 Heterologous desensitization

As a consequence of the multitude of GCPRs on human cells, many downstream signaling pathways may overlap. This poses a considerable risk for adverse effects during pharmacological treatment. In one example, the GRKs may phosphorylate not just the stimulated receptor but also intracellular domains other GCPR whereby these are rendered inactive [63-66].

1.6.3 Bone marrow egress

The bone marrow provides a sheltered niche for pluripotent HSC that give rise to both branches of the immune system and is the origin of all leukocytes in adult humans. Several studies suggest that the bone marrow niche is a highly organized structure with compartmentalization [67]. Fully mature myeloid cells are retained in the bone marrow through the interactions of bone marrow stromal cells expressing stroma derived factor 1 (SDF-1) binding chemokine receptor CXCR4. CXCR4 knockout mice display an increased number of circulating leukocytes. Stimulation of chemokine receptor 2 (CCR2) by its cognate ligand MCP-1/CCL2 cause a down regulation of CXCR4 releasing monocytes into circulation. It was previous suggested that expression of chemokines produced in proximity to peripheral inflammation could cause leukocytes to egress the bone marrow although studies have shown that the systemic levels of MCP-1 would not suffice [5]. Recent studies instead suggest that HSC in the bone marrow release MCP-1 in response to low levels of LPS causing the release of mature leukocytes [3, 4]. Similarly to the CCR2 function, CXCR2$^{-/-}$ mice display moderate bone marrow retention why an antagonistic relationship between CXCR4 and CXCR2 has been suggested [68]. CXCR2 appear to have antagonistic effects of the bone marrow retaining action of CXCR4, heterologous desensitization via upregulation of GRK3 has been suggested as a possible mechanism by which bone marrow egress is achieved [68, 69].

The human WHIM syndrome (wharts, hypogammaglobulinemi, infections and myelokatexis) is characterized by low levels of circulating leukocytes while bone marrow aspirates contain high levels of mature, mainly myeloid cells. WHIM is most commonly caused by mutations causing truncations in the cytoplasmatic tail of CXCR4 rendering it constitutively active preventing the phosphorylation necessary for receptor to internalization [70]. Interestingly, two patients with WHIM displaying wildtype *CXCR4* gene have been found to have a defect in GRK3 expression again resulting in an inability to phosphorylate the cytoplasmatic tail of CXCR4 [71].

1.6.4 Extravasation

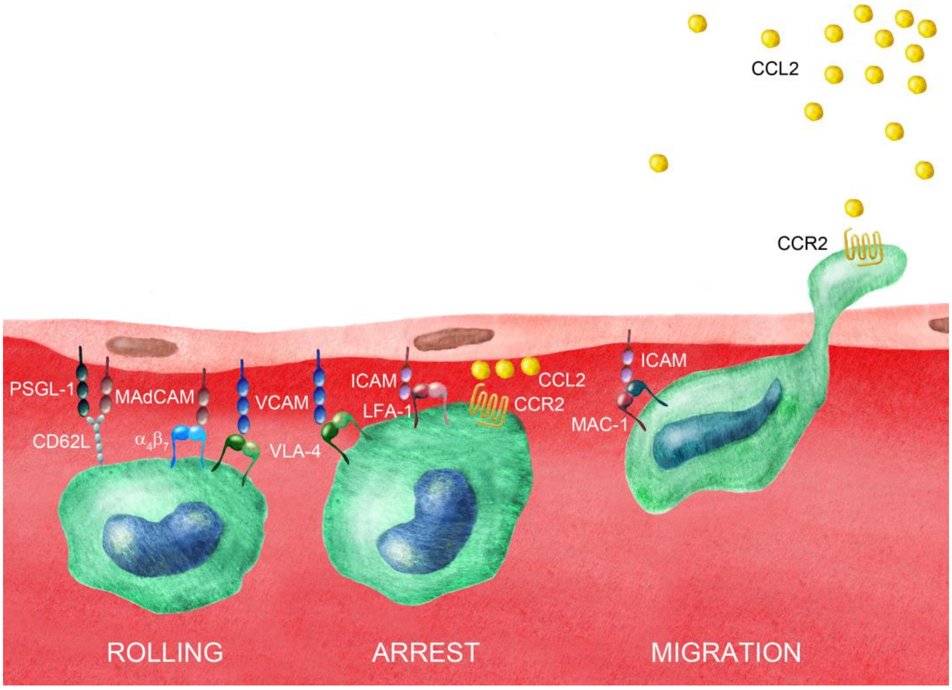

Fig 2, Schematic overview of the leukocyte adhesion cascade including slow rolling, cellular arrest and endothelial transmigration following gradient of chemokine concentration gradient. Adapted from Ley and colleagues 2007.Courtesy of Dr Malin Winerdal

Extravasation from blood vessels to tissue is initiated when sensor cells express pro-inflammatory cytokines which cause granules of preformed P-selectin in endothelial cell to fuse with the cell membrane and present on the cell surface within minutes of initiated immune response. E-selectin transcription begins simultaneously. Selectins on endothelial cells bind Lewis carbohydrate antigens like PSGL-1 on leukocytes which cause a loose association and rolling of leukocytes along the vessel due to shear flow bringing leukocytes and the endothelial surface in direct contact with each other. Intercellular adhesion molecules (ICAMs) on the endothelium may bind the leukocyte via integrins(CD11a-c:CD18) more strongly to the vessel wall. Presence of chemokines and chemokine receptors such as CCL-8 and CXCR2 cause the cytoplasmic tail of the integrin to bind the actin skeleton of the leukocyte which induce conformational changes further strengthening the adhesion bringing the cell to a stop [72]. The firm adhesion to the vessel wall is followed by extravasation where the tight cell junction in the endothelium open and the intraluminal cell begin to traverse the endothelium and continue to migrate towards the chemokine concentration gradient on stromal cells and in the (ECM).

1.6.5 Lymph node homing

The mode in which myeloid cells extravasate at sites of inflammation is very similar to the way naïve and some memory T cells enter lymph nodes. Naïve T-cells and memory T cells constantly traffic between different lymph nodes in search of their cognate antigen [73]. This is controlled by the same process as described above but the process is instead situated in the post capillary high endothelial venules (HEV) within secondary lymphoid organs such as the lymph nodes or spleen. Slow rolling is similarly induced by the lymphocyte (L-selectin, CD62L) together with ICAMs and integrins which finally cause adhesion and extravasation due to CCR7 expression on naive T-cells engaging CCL19 and or CCL21 [34]. Naïve and memory T-cells will, if they do not encounter their cognate antigen, leave the lymph node by down regulating CCR7 and instead migrate towards a gradient of S1P via the S1PR to the efferent lymphatic drainage to reenter blood circulation.

Myeloid APCs migrate through the ECM toward a concentration gradient of chemokines and eventually reach the draining lymph node via the afferent lymphatics. Both the extravasation of the T-cell and the tissue migration of the myeloid cell are guided by the chemokine CCL21 and CCL19 attracting cells expressing CCR7 [34]. Antigen presenting cells and T-cells meet in the T-cell zone of the lymph node. Here antigens are presented to naïve and memory T-cells.

Naïve B-cells, similar to T-cells need to enter the secondary lymphoid organs in order to establish the B-cell follicles. Naïve B-cell extravasate through the HEV and migrate toward a gradient of CCL13 using the CXCR5 receptor [74]. Similarly, follicular T helper cells (T_{FH}) express both CCR7 and CXCR5 enabling trafficking to B-cell follicles [75].

1.6.6 Matrix migration

Cells at the site of inflammation produce various cytokine and chemokines in the ECM which in turn diffuse in the surrounding tissue. The spread of chemokines form a concentration gradient with increasing chemokine concentration closer to the site of inflammation. How chemokine signaling cause cellular migration is still a very active research field but what is known is that the chemokines acting on chemokine receptors cause polarization of the migrating cell with active actin polymerization of the cytoskeleton in the leading edge of the cell causing protrusions in the cell membrane called lamellipodia [76]. At the other end of the cell, the actin cytoskeleton is instead degraded causing the cell to move forward.

2 AIMS OF THE THESIS

The aim of this thesis was to create an understanding of how the migratory system can be used to understand migratory function as well as phenotypic expression in different cell populations in different tissues. Additionally, the thesis aims to understand the interplay between pathogen and the immune system. The specific aims for each study are presented below.

Paper I. To investigate the levels of chemokine receptor expression on T-cells in bone marrow and peripheral blood from osteoarthritis patients and to compare the expression of these receptors with that of healthy peripheral blood.

Paper II. To investigate the presences and level of chemokine receptor expression on monocyte and monocyte precursors during their development in human bone marrow.

Paper III. To investigate the presence and function of *Aire* in myeloid DC subset and precursors in bone marrow and periphery of mice and men.

Paper IV. To investigate whether the presence of parasite infection influence serological allergy tests and if this influence would be due to the presences of the carbohydrate epitope galactose –*α-1,3*-galactose.

3 MATERIALS AND METHODS

3.1 STUDY SUBJECTS

Patients participating in studies were recruited via associated clinics. All participating study subjects were recruited after informed consent after ethical approval provided by local ethics committees according to the declaration of Helsinki.

3.1.1 Cohorts

Study subjects were included in one of the following cohorts. Patients undergoing elective arthroplastic surgery (paper I-III), patients with cat allergy or macroparasitic infection (paper IV).

3.1.2 Tissues

In papers I, II and III, human bone marrow material was obtained from the proximal femur during elective hip arthroplasty surgery. Bone marrow material was transported at room temperature in cell medium RPMI 1640 with addition of 5000IU Heparin and Streptomycin and Methicillin. Bone marrow leukocytes where extracted using homogenization, filtering, density gradient centrifugation and ACK-lysis of remaining erythrocytes

3.1.3 Healthy controls

In paper I and III, blood from healthy donors was obtained as control.

3.2 ANIMAL MODELS

Study III contain work on a rodent model. Animals were housed and handled in accordance with permission from the local ethical board.

3.2.1 AIRE deficient mice

Aire $^{-/-}$ mice were breed on C57BL/6 as previously described [40] and used together with age and sex matched littermates from heterozygote breeding as controls.

3.3 FLOW CYTOMETERY

Study I-II employed multicolor flow cytometry

3.3.1 Staining protocol

Single cells were suspended in phosphate-buffered saline with 5% autologous sera to avoid unspecific Fc-binding and stained with fluorophore coupled antibodies in different combinations. Isotype and fluorophore matched controls were used to define positive signal. Specific antibody panels can be found in the method section of respective study.

3.3.2 Sorting

Sorting of cells were carried out in paper III to analyze population separately with PCR. Sorting was carried out by magnetic sorting either manually or using AutoMACS (Miltenyi, Biotech) and/or by FACS ARIA (BD Biosciences)

3.4 PCR

Total RNA was isolated (paper III) using TRIzol reagents(Invitrogen) and cDNA was synthesized by reverse transcription. Transcripts were amplified using specific primers on CFX96 Touch(Biorad) or iCycler IQ(Biorad).

3.5 SEROLOGICAL TECHNIQUES

3.5.1 Elisa

An indirect Elisa technique was used (paper IV) to analyze levels of IgE specific for rFel d 1, cat IgA and α-Gal. A 96-well plate was coated with 0,5µg of antigen blocked with BSA. 100µl of serum was added followed by rabbit anti-human IgE followed by conjugated anti-rabbit with substrate..

3.5.2 ImmunoCAP

Total and α-Gal specific IgE were analyzed in paper IV using the ImmunoCAP System (Phadia AB, Uppsala, Sweden), according to the manufacturer's instructions.

3.5.3 Antigen blocking

Paper IV used antigen blockage with the ImmuoCAP system. This included an overnight preincubation of sera α-Gal–HAS to block IgE specific for α-Gal.

4 RESULTS AND DISCUSSION

4.1 THE EGG OR THE CHICKEN - WHAT IS OSTEOARTHROSIS?

In paper I, we address a disorder which has not previously been commonly considered to be immunologically mediated. Despite being one of the more common disorders affecting an ageing population, osteoarthrosis (OA) still remains an enigma. OA is more prevalent in weight bearing joint among the manual labour portion of the population. Thus, the notion that OA is a natural consequence of ageing is not outlandish. However, several studies have correlated OA not just to heavy labour but also to prolonged periods of physical inactivity, together with obesity and smoking. Likewise, previous trauma to the effected joint appears to be a common occurrence [77].

Unfortunately, although the mechanical "wear and tear" etiology long since has been debunked, the theory still resonates among leymen and remarkably among many physicians. Several key events in the development of OA have been established were an increased protease activity lead to diminished cartilage which in turn cause reduced cartilage shock dampening effects and increase cortical bone density.

The current treatment regime for OA consists of analgesia, physiotherapy and finally surgical replacement of the effected joint. The degradation of the joint seldom become so severe that loss of function occurs (ie. arthrodesis or dislocation). Surprisingly, the state of joint destruction poorly correlates with the pain experienced by the patient. Substantial disability due to pain often precedes the radiological findings by years. It is therefore interesting to imagine that the disease primarily could be one of altered cartilage biology with increased propensity for inflammation with increased innervation and vascularisation rather than purely a cartilage metabolic disorder.

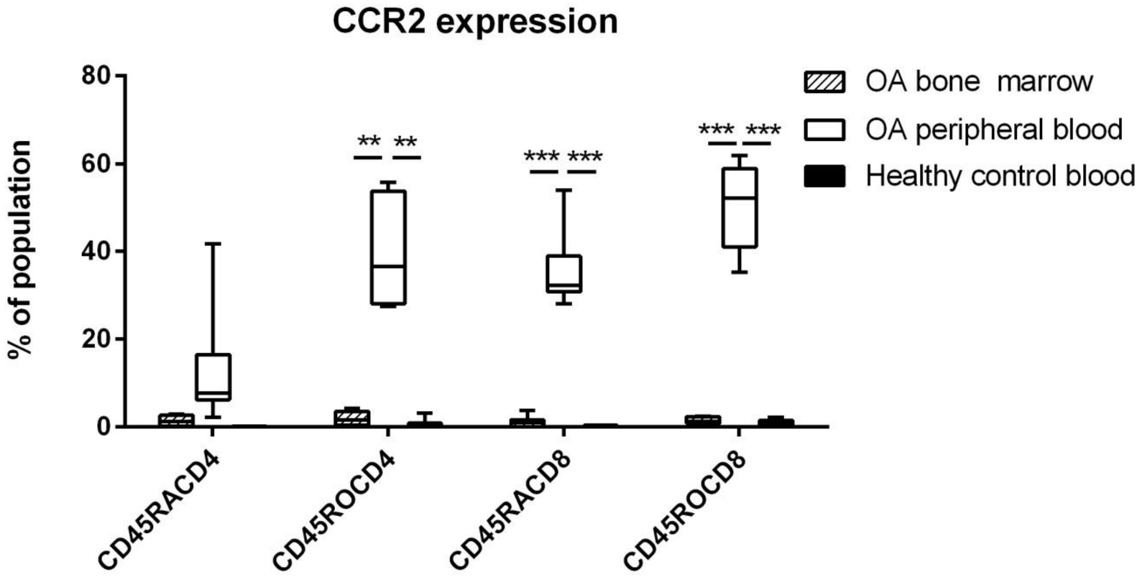

Fig 3. Percentage of memory and naïve T cells subset expressing CCR2 in peripheral blood from patients with OA (n=7) and healthy controls (n=9) together with OA bone marrow (n=7) derived T cells. Statistical analysis was performed using multiple paired t-test for OA derived patients and multiple unpaired t-test for comparison between healthy control and OA patient peripheral blood. Presented p-values are obtained using Benjamini-Hochberg correction for multiple comparisons. (p<0.05)=, (p<0.01)=**,(p<0.001)=****

Our findings in paper I suggest that patients with OA display an altered chemokine receptor function in the periphery compared to healthy controls. These findings could suggest an involvement of the adaptive immune system in disease progression although the elevated levels of CCR2 in patients OA peripheral T-cells might also suggest either a propensity of OA patients to express this chemokine alternately it could indicate an effect of pharmacological treatment [63, 64, 78]. As pain perception is central in disease pathology [79] and in the selection of patients undergoing surgery one cannot exclude the possibility that increased propensity to express CCR2 might influence pain perception. This becomes even more intriguing considering the altered pain perception of CCR2 KO mice [80, 81]. As previously demonstrated, perioperative intraarticular opioid treatment significantly reduce post-surgical pain perception despite absence of peripheral opioid receptors [82, 83].

Another aspect of these findings is the comparatively low levels of CCR2 in paired bone marrow. As we show in paper II, monocytes readily express CCR2 in bone marrow and as previously shown, also in the periphery [84]. CCR2 has been shown essential in bone marrow egress [3-5]. This could indicate that bone marrow T-cell are tissue resident without propensity to leave the bone marrow. During the study we have brushed upon several interesting and potentially useful therapeutic applications which would be of interest to investigate further. Along these lines there is a need for a deeper understanding of pharmacological effects on the often complex immune functions.

4.2 GLOBAL ASSESSMENT OF CHEMOKINE RECEPTOR EXPRESSION ON HUMAN MYELOID BONE MARROW PRECURSOR CELLS

Due to the inaccessibility of human bone marrow, studies on the early developmental stages of hematopoetic cells is often limited to animal models alternately confined to studies in peripheral blood. Since we previously demonstrated that the chemokine receptor expression of peripheral monocyte populations remains stable and with little variability during steady state, we aimed to investigate steady state conditions of commonly expressed chemokine receptors in human bone marrow on myeloid precursor cells. Previous studies have shown how fully developed myeloid cells, primarily monocytes are stored in the bone marrow and released in response to peripheral signalling[3, 4, 6]. Given the multitude of myeloid progeny it is unclear when the division between the different myeloid cell compartments types occur.

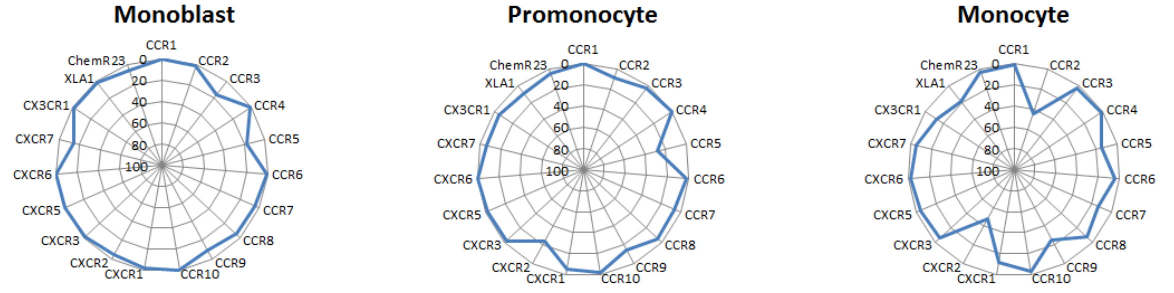

Fig 4 Spider chart displaying the average percentage of monoblasts, promonocytes and monocytes expressing of 19 chemokine receptors..

In this paper we demonstrate how several chemokine receptors associated with mature monocyte subsets are also present on early developmental stages of myeloid precursor cells. To our knowledge, we show for the first time presence of bone marrow anchoring chemokine receptor CXCR7 in non-malignant $CD34^+$ cells. Also surprising is the high prevalence of CXCR2 expressing bone marrow monocytes which in the periphery typically express CCR2. Expression of CCR2 is also present although at lower frequencies than seen in the periphery. Both CXCR2 and CCR2 has shown antagonistic function in regard to SDF-1 anchoring CXCR4 why this apparent receptor redundancy could serve to release cell upon peripheral demand.

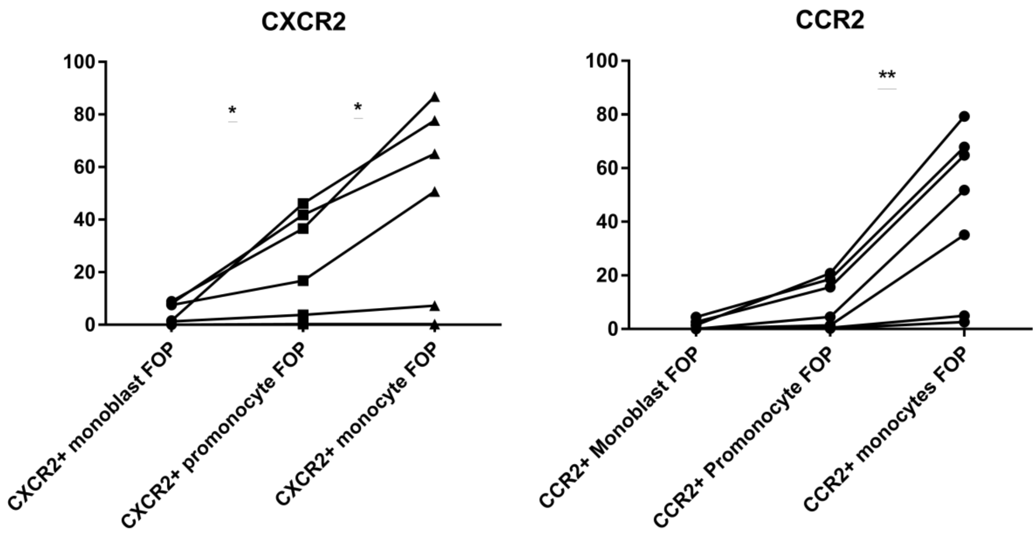

Fig 5. Percentage of monocyte and myeloid progenitor cell expressing chemokine receptor CXCR2 and CCR2 in human bone marrow. Monoblasts were $CD34^{+}$$HLA\text{-}DR^{+}$$CD14^{-}$, promonocytes $CD34^{-}$, $HLA\text{-}DR^{+}$, $CD14^{-}$ and monocytes $CD34^{-}$, $HLA\text{-}DR^{+}$, $CD14^{+}$. Statistical analysis was performed using multiple unpaired t-test. Presented p-values are obtained using Benjamini-Hochberg correction for multiple comparisons (p<0.05)=, (p<0.01)=**,(p<0.001)=****

Collectively these results forms a baseline chemokine receptor expression for myeloid precursor cell in human bone marrow and thereby provide insight into the developmental fate and characteristics of the myeloid linage and provide a comparative platform which could be used in phenotyping of myeloproliferative disorders and may aid in apheresis based treatments of chronic inflammatory disorders. The study has potential for improvement, especially additional patient samples could, in parallel to flow cytometry, be analyzed for transcriptional markers for lineage commitment. Additionally, *in vitro* culturing of sorted cells could provide insights into phenotype stability and function.

4.3 TRANSCRIPTIONAL CONTROL OF AIRE IN THE PERIPHERY IS CONTROLLED BY IFN-GAMMA AND UNDER REGULATION OF GENES IMPORTANT FOR DEVELOPMENT OF DENDRITIC CELLS IN THE BONE MARROW

In paper III, we investigate the role of the transcription factor *Aire* in myeloid bone marrow precursors. Previous studies have displayed how a subset of $33D1^+$ murine mDCs found in spleen and human $CD11c^+$ DCs in lymph nodes express the *Aire* transcription factor with a suggested function for peripheral tolerance [43]. *Aire* transcription factor has also been shown to be expressed in murine bone marrow why we wished to investigate when *Aire* expression occurred during the myeloid development of DCs. Similarly, we wished to confirm whether AIRE expression was similarly present in human bone marrow in general and in DC precursors in particular.

The pre-DC subset in murine bone marrow was shown to contain *Aire* expression, albeit at lower levels than seen in wildtype whereas AIRE expression in human pre-DC subsets from bone marrow could not be confirmed. In addition, *Aire*$^{-/-}$ deficient mice showed a diminished $33D1^+$ population but had a sustained overall DC numbers indicating a potential developmental hindrance between the pre-DC subset and the fully mature splenic $33D1^+$. We therefore investigated levels of several transcription factors including RelB, IRF4 which have been shown to be important for the development of the $33D1^+$ subset [85, 86]. Levels of RelB and IRF4 were reduced to approximately half of that of $CD8^+$control DCs whereas $33D1^+$displayed an induced expression of IRF8.

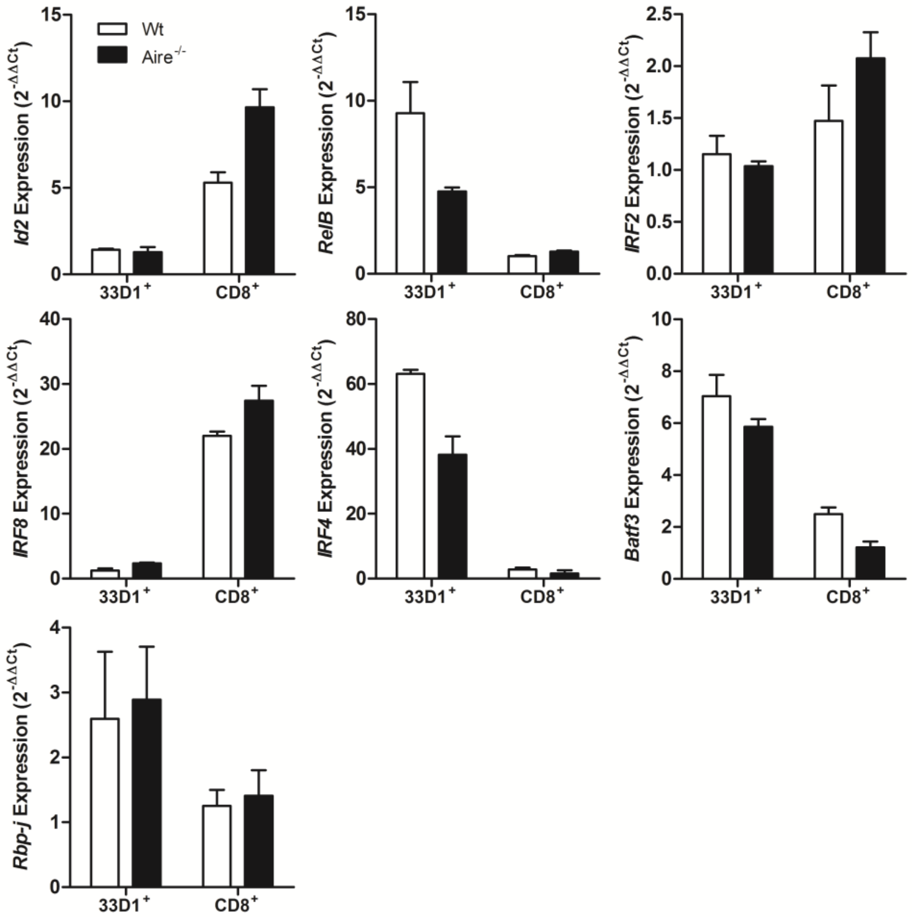

Fig 6. Relative expression of transcription factors in sorted CD8^{+} and 33D1^{+} DC subset from Aire$^{-/-}$ mice and wildtype. Bars summarize mean and standard deviation of two independent experiments.

IRF8 is also inducible by IFN-γ why wildtype 33D1^{+} DCs were stimulated with IFN-γ which abrogated *Aire* expression abruptly and remained supressed up to 14 hours . This was also seen in human peripheral myeloid DCs. Silencing of *Aire* has previously been shown to down regulate self-presentation by mTEC cells[25]. We therefore examined mRNA levels of the insulin gene *Ins2* in peripheral 33D1^{+} DCs in response to IFN-γ which as expected followed that of *Aire*. To confirm that these finding were due to IFN-γ signalling the experiment was repeated in IFN-Rγ$^{-/-}$ mice. No response was noted but instead a fivefold increase of the *Aire* expression level compared to the basal level was noted. Thus, not only self-antigens such as insulin but also Aire itself seem to be regulated by IFN-γ signalling.

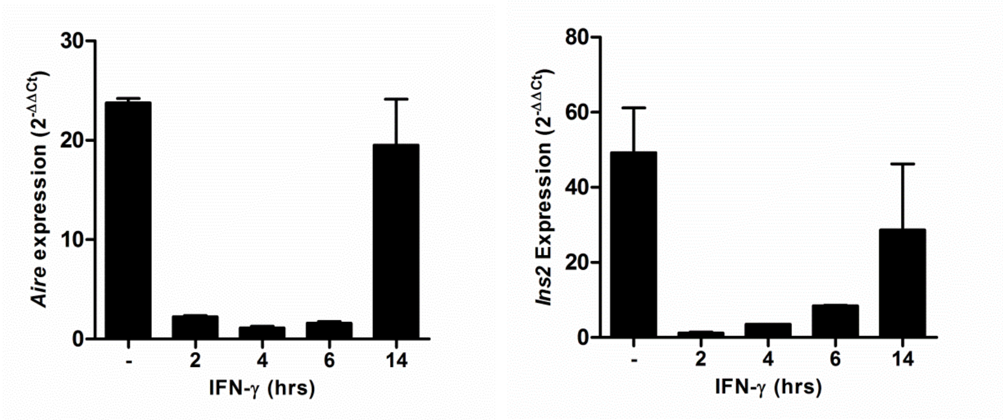

Fig 7. Relative mRNA expression of Aire and Ins2 expression measured at 2,4,6, 8 and 14 hours with qRT-PCR after stimulation of 33D1^{+} DCs with IFN-γ.

The presence of Aire expression in the periphery has previously contested [87] although an increasing body of evidence strongly suggest that Aire is expressed in the periphery and may also have functional properties[88-90]. This study show that Aire in the periphery have an impact on distribution of DC population and that TSA associates genes are down regulated in similar fashion to that of mTEC in response IFN-γ. However, it need to be further elucidated whether peripheral function of Aire impacts tolerance.

4.4 IMPACT ON IgE ALLERGY DIAGNOSTICS OF PARASITE INFECTION CAUSED BY CROSSREACTIVE IgE SPECIFIC FOR THE CARBOHYDRATE GALACTOSE-α-1, 3-GALACTOSE

Galactose-α-1,3-galactose (αGal) is a disaccharide present on some proteins as a result of posttranslational glycosylation in non-primate mammals. It is the product of the enzyme α-1,3-galactosyltranferase whose gene is abrogated in primates including humans[91]. Instead, antibodies toward αGal is widely expressed presumably due to continuous expose through the gut microbiota [92] .

The presence of preformed IgE antibodies towards the α-Gal epitope was first given clinical relevance during the introduction of Cetuximab, a monoclonal humanized mouse antibody directed toward epidermal growth factor receptor (EGFR) given to treat head and neck carcinomas. The preclinical trials which were performed in the northern USA suggested anaphylactic reactions in roughly 0.5-1% of treated patients. However, after the drug was introduced severely increased incidences of anaphylactic reactions were noted. The antigen binding domain was identified as the carbohydrate epitope Galactose-α-1,3-Galacose (α-Gal) [93, 94]. Many of the anaphylactic reaction often occurred during or shortly after the first infusion indicating the presences of preexisting antibodies. Indeed, researchers found up to a fifth of control subjects in some areas had preformed IgE antibodies to a-Gal [93]. The geographical distributions of the anaphylactic reactions suggested that some local pathogen or macroparasite caused the sensitization toward α-Gal. A high prevalence of cat allergy among patient with preformed IgE antibodies. In parallel, a new cat allergen (Fel d 5) was shown to have cross reactivity with another cat allergen were the α-Gal epitope was identified as the agent responsible for the cross reactivity [95, 96].

As these studies together suggested a potential confounding factor during allergy diagnostics based in IgE toward animal dander among parasite infected patients, paper IV examines the presence of IgE antibodies towards αGal in patients with confirmed parasite infection. In parallel, cat allergic patient are similarly confirmed to have increased titers of αGal specific IgE. Based on the IgE antibody titers we show how IgE specific for αGal and cat dander extract (CDE) as well as the α-Gal carrying allergen Fel d 5 correlate in the parasite infected patients indicating that anti-α-Gal IgE cause cross reactivity. The parasite infected patients did not however demonstrate convincing IgE titers nor titer correlation with IgE specific for the recombinant version of the major cat allergen (rFel d 1).

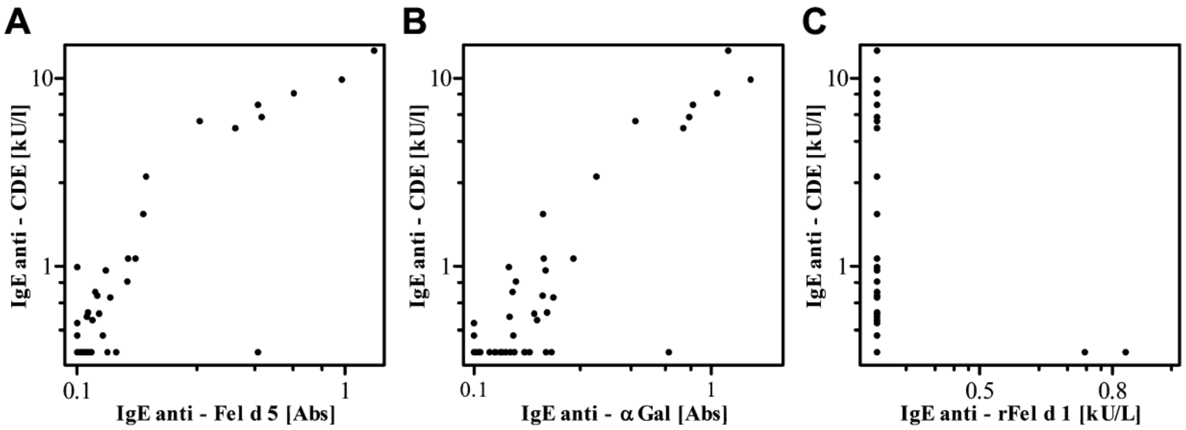

Fig 8. IgE titers among parasite infected patients (n=47) showing Spearman rank correlations between a towards cat dander extract (CDE) and A) purified cat allergen 5 (Fel d 5) (r = 0.75, P < 0.001), B) carbohydrate epitope α-Gal (r = 0.715, P < 0.001), C) recombinant cat allergen 1 (Fel d 1) (r = −0.20, P > 0.05).

Similarly, 31 cat allergic patients were examined regarding their specific IgE titers towards αGal, Fel d 5, CDE and rFel d 1. The cat allergic patients did not convincingly display correlation in specific IgE titers towards CDE and Fel d 5 nor αGal . However, the titers of IgE specific for CDE and rFel d 1correlated among the cat allergic patients indicating they unlike the parasite infected were truly sensitized to cat allergens.

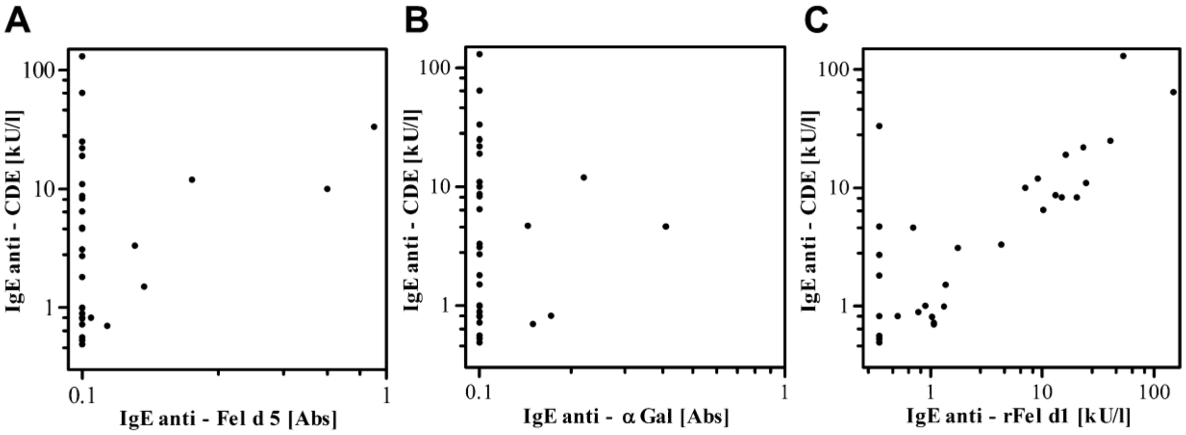

Fig 9. IgE titers among cat allergic patients (n=31) showing Spearman rank correlations between a towards cat dander extract (CDE) and A) purified cat allergen 5 (Fel d 5) (r = 0.10, P >0 .05), B) carbohydrate epitope α-Gal (r = −0.070, P >0 .05), C) recombinant cat allergen 1 (Fel d 1) (r = 0.70, P < 0.001).

To confirm that the IgE binding to CDE in the parasite infected patients were indeed due to IgE toward α-Gal we performed an inhibition assay. Sera from both patient groups were preincubated with the α-Gal epitope after which IgE titers towards CDE were assessed. As expected, the cat allergic patients still retained a high IgE titer towards CDE whereas parasite infected patients displayed a significant decrease in CDE specific IgE.

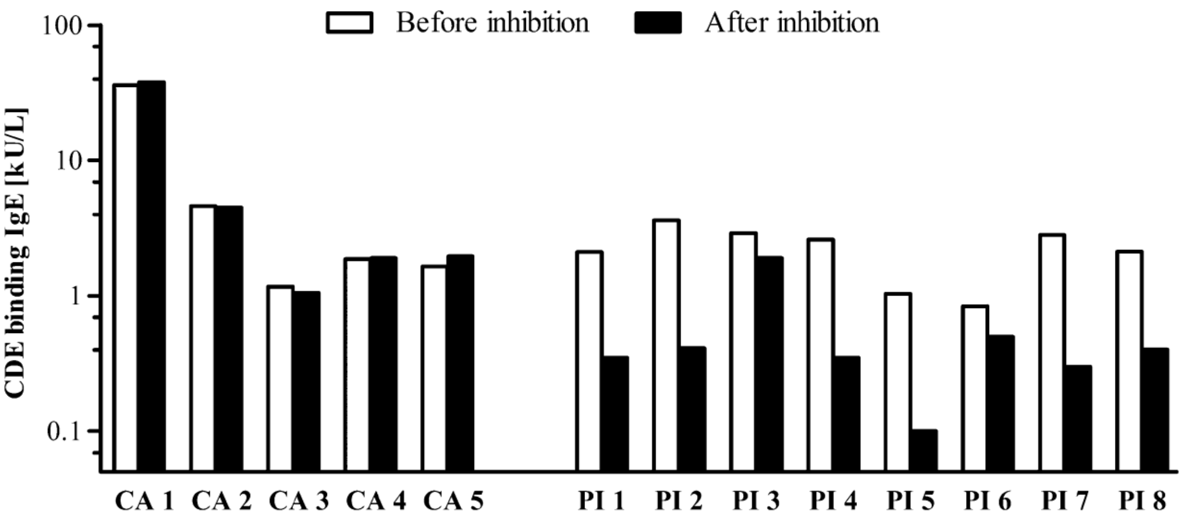

Fig 10. CDE specific IgE titers in sera from cat allergic patients (n=5) and parasite infected patients (n=8) before and after α-Gal

The use of serological analyses of allergen specific antibody titers has become widespread and is now commonly used by primary clinics as well as within veterinary medicine. Clinically, patient symptomology and history of allergic symptoms should remain the basis for diagnosis and if needed combined with skin prick test or controlled allergen challenge. However, these clinical tests are labour intensive and thereby expensive. It is therefore understandable that serological analyses have proven a useful tool for allergy diagnosis. However, serological sensitization analysis alone can be misguiding as shown in this paper and therefore need to be applied and interpreted with caution.

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Research within the field of immunology has undergone tremendous progress under the last decades. Many disorders which were previously thought to be of other origins have proven to be, at least in part, due to immunological processes. Recently, new cancer therapeutics based on immunological knowledge was granted the Nobel price. It is becoming ever more obvious that physicians in every clinical field need to deepen their knowledge of immunological processes in order to properly understand the disorders they are treating. This thesis has a wide scope concerning themes of immune migration, bone marrow development and tolerance as well as the interface between pathogens and the immune system. The study of migratory pattern in general and chemokine receptor expression in particular provide the physical backdrop and geography to immunological processes, thereby returning the field of immunology to bedside with a whole-body approach. Since the origin of immunology, most studies have been performed in peripheral blood which only provide a partial view of the underlying complexity why it is becoming ever more apparent that researcher need other sources of research material. Because of this, access to patients and patient derived material can be greatly improved trough collaboration between clinicians and researcher which benefit both researcher and clinicians and ultimately patients. This can only be achieved through an understanding of each other's work environment, potential and limitations.

In paper I we demonstrate that chemokine receptor expression in T cell populations differs between subsets and also between tissues. We were able to show that the expression of CCR2 is greatly incremented in peripheral T cells of OA patients compared to that of healthy blood donors. This is interesting given that $CCR2^{-/-}$ mice display an altered pain sensation with diminished neuropathic pain and that CCR2 display angiogenetic properties which is believed to contribute to disease progression in OA. We also prose a third line of inquiry which is that the opioids given to donors pose a potential for cross desensitization. This study therefore has several lines of inquiry which needs to be addressed in further studies.

Paper II demonstrates how chemokine receptor expression vary during myeloid bone marrow development and show a gradual increase in CXCR2 and CCR2 which both have been shown to possess a switch function capable of releasing myeloid cells into the circulation upon demand. We demonstrate CXCR7 expression in the monoblast stage which to our knowledge is the first non-malignant cell type to do so. The study still has room for improvement and could greatly benefit additional comparative material as well as confirming analytical methods.

Paper III confirms that *Aire* is expressed in peripheral 33D1 DCs. Furthermore, *Aire* expression was present in bone marrow from mice but we were unable to confirm AIRE expression in cells from human bone marrow. Based on findings of altered peripheral DC composition in $Aire^{-/-}$mice we performed a series of stimulation studies suggesting that the transcription factor RelB is under control of Aire. We also show how *Aire* and related self-antigen gene *Ins2* are down regulated in response to the inflammatory cytokine IFN-γ.

In Paper IV we address the commonality of IgE responses between macroparasite infections and allergy. We demonstrate how macroparasitic infections influence the serology of patients which renders them sensitized to cat dander. We also show that the cross reactivity is dependent on the carbohydrate αGal. We display how true cat allergic patients can be distinguished from patients with macroparasitic infections using a component resolution based serology analysis.

This study has been followed by others suggesting that the sensitization toward αGal could be achieved through tick bite and that sensitization to αGal may result in red meat allergy. The serology aspect is interesting although no substantial effort has been made to understand how IgE production towards an antigen is achieved which could present an interesting line of research. Also, the field of parasitology has due to it nature as third world disorder not gained as much interest nor funding as it otherwise should. Since macroparasites truly are masters of their environment it would be of great interest to further understand how they avoid immune detection and as in some cases gain tolerance from the immune system.

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